PCT

(51) International Patent Classification 5 : C07K 7/10	A1
(11) International Publication Number: WO 93/15110
(43) International Publication Date: 5 August 1993 (05.08.93)

(21) International Application Number: PCT/US93/00884

(22) International Filing Date: 1 February 1993 (01.02.93)

(30) Priority data:
829,140   31 January 1992 (31.01.92)   US

(71) Applicant: WHITEHEAD INSTITUTE FOR BIOMEDI-CAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US).

(72) Inventors: O'SHEA, Erin. K. ; 37 Country Corners Road, Wayland, MA 01778 (US). KIM, Peter, S. ; 4 Middleby Road, Lexington, MA 02173 (US).

(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

C07K 14/00B

BEST AVAILABLE COPY

(54) Title: AMINO ACID SEQUENCES WHICH PAIR SPECIFICALLY
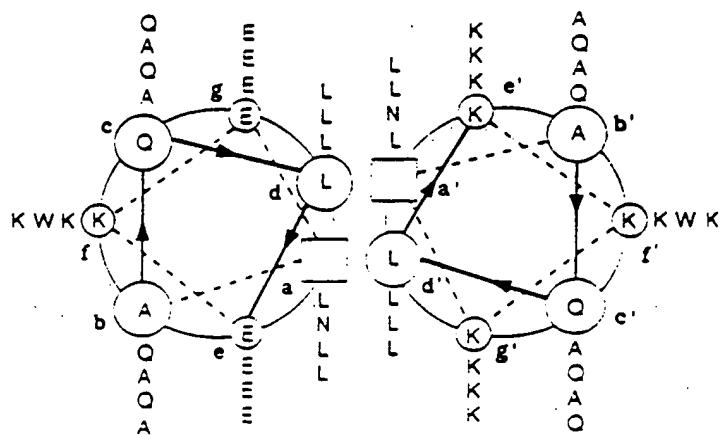
ACID-p1   BASE-p1

(57) Abstract

Pairs of synthetic peptides designed in such a manner that they pair specifically with one another to form a heterodimer and then, once paired, preferentially fold as a helical heterodimer. The peptide members of the pair may be of any length, provided they are sufficiently long that they are stable in the heterodimeric form and are able to fold into a helical configuration. The synthetic peptide pairs of the present invention can be used as affinity reagents to isolate associated molecules. In addition, because the heterodimer is preferentially formed and very stable under physiological conditions, the synthetic peptides of the present invention are suitable for use *in vivo*. The synthetic peptide pairs can be used, for example, for *in vivo* applications in which two molecules or components necessary for a given event must be brought together for the event to occur. The synthetic peptide pairs of the present invention make it possible to bring the components together with great specificity and affinity. Alternatively, the synthetic peptide pairs can be designed to prevent binding or interaction of molecules (e.g., DNA and its DNA binding protein) necessary for an event to occur. The synthetic peptide pairs can also be used as a biodegradable molecular velcro, such as in grafting and for artificial sutures.

AMINO ACID SEQUENCES WHICH PAIR
SPECIFICALLY

Description

Background

5   Specificity in protein-protein interactions is not
well understood, although considerable effort has been
expended in trying to explain what determines such inter-
actions. It would be very use...1 to know what character-
istics of an amino acid sequence contribute to protein-
10 protein interactions.

Summary of the Invention

       The present invention relates to pairs of synthetic
peptides designed in such a manner that they pair specifi-
cally with one another to form a heterodimer and then,
15 once paired, preferentially fold as a helical heterodimer.
The present invention further relates to a method of
making pairs of synthetic peptides which bind prefer-
entially to one another and to methods of producing such
peptide pairs. The peptide members of the pair may be of
20 any length, provided they are sufficiently long that they
are stable in the heterodimeric form and are able to fold
into a helical configuration. The two members of a pair
will typically be of the same length, although this is not
required. In general, the individual peptides will be at
25 least 6 to 8 amino acid residues in length, generally at
least 12-14 amino acid residues in length and will prefer-
ably be at least 16-20 amino acid residues in length. In
one embodiment, the individual peptides will be at least
20-23 amino acids in length. In another embodiment, the
30 individual peptides will be 24-30 amino acids in length,
particularly to 30 amino acids in length. There is no

-2-

upper limit on individual peptide length. More than one
peptide "repeat" or unit may be combined in a peptide pair
of the present invention, if desired. That is, more than
one peptide which pairs specifically with another peptide
5   to form a heterodimer and, once paired, forms a coiled-
coil helical heterodimer can be present in a synthetic
peptide of the present invention; the second member of the
peptide pair also includes multiple peptide repeats or
units, whose amino acid sequences are designed to pair
10   specifically and avidly with the first peptide repeats and
preferentially form a coiled-coil helical heterodimer. In
particular, it relates to pairs of synthetic peptides
(whose members are designated ACID-p1 and BASE-p1 herein
and A-1 and B-1 in U.S. Application, Serial No.
15   07/829,140, to which the subject application claims prior-
ity) in which the amino acid sequence has been designed in
such a manner that the two members of the pair "stick" to
each other with great specificity and avidity. In con-
trast, neither peptide sticks efficiently to itself. The
20   amino acid residues which comprise the members of the
peptide pairs can be naturally-occurring amino acid resi-
dues, non-naturally occurring amino acid residues or
modified amino acid residues. As described herein, under
physiological conditions of temperature and pH, homodimers
25   of the synthetic peptides are very unstable, relative to
heterodimers of the synthetic peptides and, in an equil-
ibrium mixture of the two peptides, the heterodimer is
favored over the homodimers by at least 1-million-fold.
In one embodiment of the present invention, two
30   peptides (designated A-1 and B-1 in U.S. Application,
Serial No. 07/829,140) designed to bind one another spec-
ifically and avidly and to preferentially form a helical
heterodimer have been synth sized and characterized, using
known methods. As described herein, under physiological
35   conditions, individual peptides have been shown, using

-3-

circular dichroism (CD) spectroscopy, to be predominantly
unfolded in isolation and when combined, to associate
preferentially to produce stable, parallel, coiled-coil
(helical) heterodimers. Further, the degree of preference
5 for the heterodimers has been estimated by studying the
stability of the heterodimers and homodimers, using CD
methods; results showed that the heterodimer has much
greater stability than either of the homodimers. The
observed difference in stability between the homodimers
10 and the heterodimer suggests that the heterodimer is
preferred over the homodimers by at least 1-million-fold.
In addition, the oligomerization state, helical content
and helix orientation can be assessed, using, respective-
ly, sedimentation equilibrium studies, CD and disulfide
15 bonding of the peptides in the desired parallel orient-
ation, followed by measurement of the concentration depen-
dence of stability.

    In a specific embodiment of the present invention,
two peptides, each containing two and 14 amino acid re-
20 peats designated, respectively, ACID-p1 and BASE-p1,
(designated A-1 and B-1 in a related U.S. Application,
Serial No. 07/829,140) were designed to bind to one
another specifically and avidly and to preferentially form
a helical heterodimer. The two peptides have been syn-
25 thesized and characterized using known methods. Using the
same methods as described above and further described
herein, under physiological conditions, the individual
peptides (ACID-p1 and BASE-p1) were shown to be pre-
dominantly unfolded and an equimolar mixture of ACID-p1
30 and BASE-p1 has been shown to preferentially form hetero-
dimers which are stable, parallel in orientation and
highly helical. The heterodimer has been shown to have
greater stability than either of the homodimers. The
amino acid composition and order of the constituent pep-
35 tides were designed to introduce destabilizing electro-

static interactions in the homodimers that would be re-
lieved in the heterodimers. The two peptides differ only
at two positions (designated e and g, as defined below) by
a single amino acid. The ACID-p1 peptide contains, at

5   these two positions, an acidic amino acid, such as glu-
tamic acid; the BASE-p1 peptide contains, at these two
positions, a basic amino acid, such as lysine. Measure-
ments of the specificity of dimer formation demonstrate
that these peptides have at least 10^5-fold preference for

10 the heterodimeric state. Studies of the pH and ionic
—   strength dependence of stability confirm that electro-
static destabilization of the homodimers provides the
primary driving force for the specificity of heterodimer
formation. Finally, the oligomerization state, helical

15 content and helix orientation were assessed using respec-
tively, sedimentation equilibrium studies, CD and di-
sulfide bonding of the peptides in the desired parallel
orientation, followed by measurement of the concentration
dependence stability.

20   The synthetic peptide pairs of the present invention
can be used as affinity reagents to isolate associated
molecules. For example, a synthetic peptide pair, such as
ACID-p1 and BASE-p1, can be used in place of biotin-avidin
(e.g., in a biotin/streptavidin affinity method), epitope

25 tagging or immunoaffinity purification methodology. In
addition, because the heterodimer is preferentially formed
and very stable under physiological conditions, the syn-
thetic peptides of the present invention are suitable for
use in vivo. In this embodiment, for example, one member

30 of the synthetic peptide pair (e.g., ACID-p1) can be
produced as part of a chimeric or hybrid peptide product
(chimeric peptide 1), which includes the synthetic peptide
(one member of the peptide pair) and an additional com-
ponent which is not a synthetic peptide of the pr sent

35 invention. The second component can be a peptide, poly-

-5-

peptide, glyco- or other protein, a detectable label or a
small organic molecule, which is to be joined or brought
into contact with another molecule. The other member of
the synthetic peptide pair can be, for example, included
5   in a second chimeric or hybrid product (chimeric peptide
2) which includes the synthetic peptide (e.g., BASE-p1)
and the molecule with which the additional component of
the first chimeric product (chimeric peptide 1) is to bind
or otherwise interact with. Such chimeric or fusion
10 peptides are also the subject of this invention. They can
be produced using known techniques, such as recombinant
production methods or chemical (synthetic) methods. They
can be produced as one product (e.g., a chimeric peptide
which includes both components as produced) or the com-
15 ponents can be produced individu. ly and then joined,
using known methods. The synthetic peptide pairs can be
used, for example, for in vivo applications in which two
molecules or components necessary for a given event must
be brought together for the event to occur. The synthetic
20 peptide pairs of the present invention make it possible to
bring the components together with great specificity and
affinity. Alternatively, the synthetic peptide pairs can
be designed to prevent binding or interaction of molecules
(e.g., DNA and its DNA binding protein) necessary for an
25 event to occur. The synthetic peptide pairs can also be
used for biodegradable procedures involving grafting and
artificial sutures.

Brief Description of the Drawings
       Figure 1 is a coiled-coil helical wheel represen-
30 tation of amino acid residues present in peptide A and
peptide B at the locations indicated.
       Figure 2 is a coiled-coil wheel representation of the
sequence of the ACID-p1/ACID-p1 peptide homodimer (Panel
A), the BASE-p1/BASE-p1 peptide homodimer (Panel B) and

-6-

the ACID-p1/BASE-p1 peptide heterodimer of the present
invention (Panel C). In this representation, the sequence
of the peptide ACID-p1 and peptide BASE-p1 is arrayed on a
coiled-coil diagram in which the helices of the dimer are
5   viewed from the N-terminus (with the helix axis projecting
into the page). The sequence of the coiled-coil peptide
is divided into positions of the heptad repeat, labeled a-
g; amino acid residues at positions a and d make up the 4-
3 hydrophobic repeat characteristic of coiled coils.
10   Amino acids are represented by their respective one-letter
code.

Figure 3 presents circular dichroism spectra of the
ACID-p1 peptide, the BASE-p1 peptide and the ACID-p1/BASE-
p1 heterodimer at 37°C in phosphate buffered saline (PBS),
15   pH 7.0 (physiological conditions).

Figure 4 presents a circular dichroism melting curve
of the ACID-p1/BASE-p1 heterodimer at a wavelength of
222nm.

Detailed Description of the Invention

20   The present invention is based on Applicant's dis-
covery of characteristics of pairs of peptides necessary
and sufficient for such peptides to bind or pair specific-
ally and avidly to one another, to form heterodimers, and
for the resulting heterodimers to preferentially fold as a
25   helical heterodimer. As a result of this discovery,
Applicant has designed and produced peptide pair members
which, when mixed, associate preferentially to form a
stable, parallel coiled-coil heterodimer, rather than
their respective homodimeric forms. The peptides of the
30   present invention may be of any length, provided that they
are sufficiently long to be stable when paired with the
other member of the peptide pair (i.e., in the hetero-
dimeric form) and to be able to allow the heterodimer to
fold into a helical configuration. Generally, the pep-

-7-

tides will be at least 6 to 8 amino acid residues, gener-
ally at least 12 to 14 amino acid residues in length and
preferably, at least 16 to 20 amino acid residues in
length or between 20 or 30 amino acid residues long. In
5 one embodiment, each member of the peptide pair has two 14
amino acid residue "repeats" (two 14 amino acid units)
resulting in each pair member being 28 amino acids long.
In another embodiment, each pair member is 30 amino acids
long. In another embodiment, each pair member is 100
10 amino acids long. There is no upper limit on the length
of peptide members and the appropriate length will be
determined by such considerations as the context in which
a peptide pair is to be used (e.g., _in vitro_ or _in vivo_,
isolated method, therapeutic or diagnostic techniques).
15 More than one peptide repeats or units of the present
invention can be combined (i.e., present in a single
multi-unit peptide) if desired. For example, more than
one repeat peptide designed to pair specifically and
avidly to a second peptide, as described herein (e.g,
20 designated peptide ACID-p1 herein and A-1 in U.S. Appli-
cation Serial No. 07/829,140, to which the subject appli-
cation claims priority), can be combined in a first multi-
unit peptide and more than one repeat of the second pep-
tide, (e.g., designated peptide BASE-p1 herein and B-1 in
25 U.S. Application Serial No. 07/829,140) also described
herein, can be combined in a second multi-unit peptide and
the two multi-unit peptides used as the two members of the
peptide pair. The individual peptide units in the multi-
unit peptide can be the same or different. In one embodi-
30 ment described herein, the individual peptide units differ
within a peptide (e.g., there are two or more peptide
units which are not identical in sequence) but are the
same between peptides (e.g., the two members of the pep-
tide pair each contain the same peptide unit(s) as the
35 other member). Amino acids in the peptides can be natu-

-8-

rally-occurring amino acids, non-naturally-occurring amino
acids or modified amino acids. The members of a peptide
pair stick avidly to the other member of the pair, but do
not bind efficiently to a like member of the pair. The
5   following is a description of the rationale underlying the
design of the peptide pairs; design synthesis and charac-
teristics of peptide pairs, particularly of peptides ACID-
pl and BASE-pl, of the present invention; and uses there-
for.

10   Figures 1 and 2 are coiled-coil helical wheel repre-
sentations of 30 amino acid residues of the ACID-pl/ACID-
pl peptide homodimer (Figure 2, Panel A), the BASE-Pl/
BASE-Pl peptide homodimer (Figure 2, Panel B) and the
ACID-pl/BASE-pl peptide heterodimer (Figure 2, Panel C).
15 The sequence of the coiled-coil peptide represented is
divided into positions of the heptad repeat, which are
labeled a through g. Selection of the appropriate type of
amino acid residue for each of the positions relied on
studies of naturally-occurring leucine zipper peptides,
20 particularly the Fos/Jun leucine zipper heterodimer and
the GCN4 leucine zipper homodimer, as described below.

The peptide pairs described herein were designed
based on principles learned from Applicant's study of
coiled-coil amino acid sequences, including the hetero-
25 dimeric Fos/Jun leucine zipper. The peptide pairs are
designed in such a manner that, in isolation, the indi-
vidual peptides are unfolded and do not join or stick to a
like peptide efficiently (i.e., two of the same peptide
pair members do not stick efficiently together to form
30 homodimers). In contrast, when mixed together, the pep-
tide pair members join preferentially to form a stable
heterodimeric coiled-coil. The primary driving force for
the specificity of binding to form a heterodimer is elec-
trostatic destabilization of the homodimers. As shown by
35 results presented herein, the designed peptides fold as

-9-

parallel, helical dimers which have a great (at least about 10^5-fold) preference for the heterodimeric state.

Previous work had demonstrated that an approximately 35 amino acid leucine zipper region from Fos and from Jun

5 is necessary for preferential heterodimer formation. Landschulz, W.H. et. al., Science, 240:1759-1764 (1988); Ransone, L.J. and I.M. Verma, Ann. Rev. Cell Biol. 6:539-557 (1990). Studies of the Fos and Jun leucine zipper heterodimer, as described in Example 1, showed that eight

10 residues at positions e and g of the coiled-coil peptide (heptad repeat) are the primary determinants of specific-ity of pairing of the two peptides (Fos and Jun) to form a heterodimer. As described in Example 1, 8 residues from Fos and from Jun, in the background of the GCN4 leucine

15 zipper, have been shown to be sufficient to mediate pre-ferential heterodimer formation. Amino acid residues at all other positions of the heptad can be substituted without significant loss of specificity of Fos/Jun pair-ing. Residues present at positions e and g in the Fos

20 peptide ar- predominantly acidic. There are many basic residues ; positions e and g in the Jun peptide. Studies c: the pH dependence of stability suggest that, at neutral pH, Fos is destabilized by these acidic residues and Jun is slightly destabilized by basic residues. Under the

25 same conditions of pH, the Fos/Jun heterodimer has greater stability, due to relief of destabilizing interhelical electrostatic interactions and potential ion pairs.

Examination of the x-ray structure of the GCN4 leu-cine zipper shows that amino acid residues at positions e

30 and g' (and g and e') are close in space. The residues present at these positions are of opposite charge in the GCN4 homodimer and are seen to participate in interhelical ion pairs. O'Shea, E.K. et al., Science, 254:539-544 (1991).

-10-

Relying on the above-described information about the
Fos/Jun leucine zipper and the GCN4 leucine zipper, pairs
of peptides which pair specifically and avidly to one
another and subsequently preferentially form a helical
5 heterodimer have been designed and these peptides have
been synthesized and characterized.

In the peptide pairs of the present invention,
charged amino acid residues are present at positions e and
g (and e' and g'; Figure 1). In one peptide (in Figure 1,
10 designated peptide ACID-p1), a negatively charged amino
acid, such as glutamate (glutamic acid), is present and in
the second peptide (in Figure 1, designated peptide BASE-
p1), a positively charged amino acid, such as lysine, is
present. As shown in Figure 1, a negatively charged amino
15 acid residue is present at e (amino acid residues 4, 11,
18, 25) and g (amino acid residues 6, 13, 20, 27) in
peptide ACID-p1 and a positively charged amino acid is
present at e' (amino acid residues 4, 11, 21, 25) and g'
(amino acid residues 6, 13, 20, 27) in peptide BASE-p1. A
20 hydrophobic amino acid, such as leucine, is present at
positions a and d (peptide ACID-p1) and a' and d' (peptide
BASE-p1). Leucine is a preferred choice because it is the
most common amino acid at these positions in naturally-
occurring coiled coils. However, not all amino acid
25 residues at these positions must be hydrophobic; there may
be other (non-hydrophobic) amino acids present at these
positions, provided that those present at these positions
in aggregate are predominantly hydrophobic. In deter-
mining appropriate residues for these positions, reference
30 can be made to work by Hodges and co-workers insert
(Hodges, R.S. et al., J. Biol. Chem., 256:1214-1224
(1981); Hodges, R.S. et al., Peptide Res., 1:19-30 (1988);
Lau, S.Y. et al., J. Biol. Chem., 259:13253-13261 (1984))
and Conway and Parry ((Int. J. Biol. Macromol., 12:328-334
35 (1990) and Example 1. In one embodiment of the present

invention, an asparagine is present at the second a posi-
tion (in peptide ACID-p1, position a and in peptide BASE-
p1, position a', both of which represent amino acid 14) in
order to favor the parallel orientation and discourage

5   higher order oligomerization. Asparagine is present at
the corresponding position in the GCN4 leucine zipper,
which is a peptide which folds as a two-stranded parallel
coiled coil. The amino acid residues present at positions
b, c and f, as represented in Figure 1 can be very varied;

10 almost any combination of amino acid residues can be used,
provided that there is an appropriate distribution of
hydrophilic amino acid residues at these positions.
Selection of amino acid residues appropriate for inclusion
at positions b, c and f can be made with reference to

15 Applicants' work (e.g., Example 1) and work by Conway and
Parry (see above).

      In one embodiment of the present invention, as repre-
sented in Figure 2, small, uncharged amino acid residues,
particularly those which are good helix formers, are

20 present at positions b and c and b' and c'. Such small,
uncharged amino acid residues as alanine and glutamine are
used at these locations in order to prevent residues at b
and c and b' and c' from interacting with residues at
positions e and g and e' and g' and, as a result, com-

25 peting with the desired interhelical interactions. In
addition, polar residues included in the peptide pair
members increase solubility of the peptide. In this
embodiment, charged residues, such as glutamate and ly-
sine, are included at position f (and f', see Figure 1)

30 because many charged residues are found at this location
in naturally-occurring coiled coils. These residues also
serve to increase solubility and discourage higher-order
oligomerization. A single tryptophan at position f and f'
has been included in peptides ACID-p1 and BASE-P1 as a

35 means of facilitating concentration determination by

-12-

absorbance. However, they will generally be a charged
residue, such as glutamate or lysine.

Thus, the two members (peptide ACID-p1 and peptide
BASE-p1) of the synthetic peptide pairs of the present
5   invention have been a general formula which can be des-
cribed with reference to the coiled-coil helical wheel
representation shown in Figure 1. The following amino
acid residues are present:

In a peptide A

10   1.   at positions e and g, negatively charged amino acids
          residues, such as glutamate (glutamic acid);

     2.   at positions a and d, a hydrophobic amino acid res-
          idue, such as leucine; and

     3.   at positions b, c and f, almost any amino acid res-
15   idues, provided that there is an appropriate distri-
          bution of hydrophilic amino acids, such as a nega-
          tively charged amino acid (e.g., alanine, glutamine)
          at b and c and such as a positively charged amino
          residue (e.g., glutamate, lysine) at f.

20   In peptide B

     1.   at positions e' and g', positively charged amino acid
          residues, such as lysine;

     2.   at positions a' and d', a hydrophobic amino acid
          residue, such as leucine; and

25   3.   at positions b', c' and f', almost any amino acid
          residues, provided that there is an appropriate
          distribution of hydrophilic amino acids, such as a
          negatively charged amino acid residue (e.g., alanine,
          glutamine) at b' and c' and such as a positively
30   charged amino acid residue (e.g., glutamate, lysine)
          at f'. In general, proline will not be used in these
          peptides.

-13-

The amino acid residues present at each of these
positions in one embodiment of the two pair members are
shown in Figure 2 (by their one-letter code) and in Table
1 (peptide ACID-p1, peptide BASE-p1, respectively, by
5 their three-letter codes).

-14-

TABLE 1

AMINO ACID SEQUENCE OF PEPTIDE ACID-p1 AND BASE-p1

Peptide ACID-p1			Peptide BASE-p1		
	1	Ala (A)*		1	Ala (A)
5	2	Gln (Q)		2	Gln (Q)
	3	Leu (L)		3	Leu (L)
	4	Glu (E)		4	Lys (K)
	5	Lys (K)		5	Lys (K)
	6	Glu (E)		6	Lys (K)
10	7	Leu (L)		7	Leu (L)
	8	Gln (Q)		8	Gln (Q)
—	9	Ala (A)		9	Ala (A)
	10	Leu (L)		10	Leu (L)
	11	Glu (E)		11	Lys (K)
15	12	Lys (K)		12	Lys (K)
	13	Glu (E)		13	Lys (K)
	14	Asn (N)		14	Asn (N)
	15	Ala (A)		15	Ala (A)
	16	Gln (Q)		16	Gln (Q)
20	17	Leu (L)		17	Leu (L)
	18	Glu (E)		18	Lys (K)
	19	Trp (W)		19	Trp (W)
	20	Glu (E)		20	Lys (K)
	21	Leu (L)		21	Leu (L)
25	22	Gln (Q)		22	Gln (Q)
	23	Ala (A)		23	Ala (A)
	24	Leu (L)		24	Leu (L)
	25	Glu (E)		25	Lys (K)
	26	Lys (K)		26	Lys (K)
30	27	Glu (E)		27	Lys (K)
	28	Leu (L)		28	Leu (L)
	29	Ala (A)		29	Ala (A)
	30	Gln (Q)		30	Gln (Q)

*One letter amino acid symbol.

35   Figure 2, the amino acid residues present at posi-
tions a through g of peptide ACID-1 in the heterodimer
(Panel C) correspond to amino acid residues 1 through 30
for peptide ACID-p1 in Table 1 and as shown in Table 2.

-15-

TABLE 2

Amino Acid residue	Position in Figure 2	Position in Table 1
Leu	a	7
5 Asn		14
Leu		21
Leu		28
Ala	b	1
Gln		8
10 Ala		15
Gln		22
— Ala		29
Gln	c	2
Ala		9
15 Gln		16
Ala		23
Gln		30
Leu	d	3
Leu		10
20 Leu		17
Leu		24
Glu	e	4
Glu		11
Glu		18
25 Glu		25
Glu	f	5
Lys		12
Trp		19
Lys		26
30 Glu	g	6
Glu		13
Glu		20
Glu		27

In Figure 2, the amino acid residues present at
35 positions a' through g' of peptide BASE-pl in the hetero-
dimer (Panel C) correspond to amino acid residues 1
through 30 for peptide BASE-pl in Table 1 and as shown in
Table 2.

-16-

TABLE 3

Amino Acid residue	Position in Figure 2	Position in Table 1
Leu	a'	7
5 Asn		14
Leu		21
Leu		28
Ala	b'	1
Gln		8
10 Ala		15
Gln		22
Ala		29
Gln	c'	2
Ala		9
15 Gln		16
Ala		23
Gln		30
Leu	d'	3
Leu		10
20 Leu		17
Leu		24
Lys	e'	4
Lys		11
Lys		18
25 Lys		25
Glu	f'	5
Lys		12
Leu		19
Glu		26
30 Lys	g'	6
Lys		13
Lys		20
Lys		27

The amino acid residues can additionally include Cys-Gly-
35 Gly, which is generally added at the N-terminal of the
peptide and not part of the 30 amino acid residue peptide
of the present invention but, rather, is included for
assay purposes only. For example, Cys-Gly-Gly can be
added to the peptide (before amino acid residue 1) to

-17-

assist in the assessment of helix orientation (parallel vs. antiparallel) of the heterodimer. To do this, a disulfide-bonded peptide (disulfide-bonded in parallel orientation) is assessed by measuring its stability as a

5   function of peptide concentration; alternatively, molecular weight can be measured as a function of peptide concentration by sedimentation. If the helices are parallel, the disulfide-bonded peptide is expected to have stability independent of peptide concentration and molecular weight

10   equal to the molecular weight of the dimer independent of peptide concentration.

       Other short peptide pairs which pair specifically and avidly and subsequently preferentially form helical heterodimers can be produced, using the teachings of the sub-

15   ject application as to the appropriate amino acid residues and known synthetic methods. Once a peptide pair has been produced, its ability to pair specifically and avidly and to preferentially form helical heterodimers can be assessed, as described herein for peptide ACID-p1 and pep-

20   tide BASE-p1, using known methods.

       The members of the peptide pairs of the present invention can be produced using known methods, such as chemical synthesis or recombinant/genetic engineering technology. For example, they can be synthesized as

25   described in Example 2 or in much the same manner as described in Example 1 for the synthesis of the Fos and Jun leucine zipper peptides. Alternatively, the peptides can be produced in an appropriate host cell by expressing DNA or RNA encoding the peptide sequence. As used herein,

30   the term synthetic refers to peptides of the present invention made by any method (e.g., chemically or by recombinant or genetic engineering methods). Peptide pairs of the present invention have many uses, in both _in vitro_ and _in vivo_ contexts. For example, peptide pairs

35   can be used as affinity reagents, in much the same way as

-18-

or as a replacement for other binding pairs. For example,
they can be used in place of biotin-streptavidin, epitope
tagging methods or immunoseparation methods. In this use,
a member of a peptide pair can be attached or linked to
5   another molecule (e.g., another peptide, polypeptide,
glyco- or other protein, or a detectable label or small
organic molecule) or to a solid support (e.g., a column,
particle, filter, plastic plate) by known methods, such as
a component of a fusion protein or through a linker (e.g.,
10 the Cys/Gly/Gly referred to above) for attachment to a
solid surface. A molecule to which the second member of
the peptide pair is attached (e.g., by chemical or recom-
binant methods) can be separated or isolated by contacting
a mixture containing the molecule-peptide pair with the
15 solid surface bearing the second member of the peptide
pair, under conditions appropriate for sticking or pairing
of the peptide pair members. The fraction of the mixture
which is not the molecule to be separated or isolated will
not become affixed to the solid support and can be removed
20 simply by separating the solid support from the remainder
of the mixture. The bound molecule (bound as a result of
pairing of the peptide pair members) can be released from
the solid support by, for example, changing the pH and/or
temperature of the bound fraction. Such a method of
25 separating or isolating a molecule in this manner can be
used, for example, for purification of a molecule to be
used for other purposes (e.g., where presence of a par-
ticular substance is indicative of the presence or absence
of a disease or condition). Peptide pairs of the present
30 method can also be use for in vivo purposes, such as to
block, induce or enhance an event in cells (e.g., to
interfere with binding of two components in a cell where
binding is necessary, thus inducing or enhancing the
event). In this embodiment, peptides of the present
35 invention can be produced in cells in which they are to

-19-

act by, for example, expression from a vector, such as a
retroviral vector(s) containing DNA or RNA encoding a
chimeric peptide or peptides. The chimeric peptide in-
cludes the amino acid residues of the peptide of the
5   present invention and, if desired, a peptide or a poly-
peptide which is not a synthetic peptide of the present
invention, such as a peptide which is to act in the cell.
Alternatively, peptides of the present invention can be
used for radioimaging or to treat diseases, such as malig-
10   nancies. In the case of malignancies, one member of a
peptide pair is expressed in the malignant cell (e.g.,
from an appropriate vector). The second member of the
peptide pair can be labeled, thereby capable of detection
or can be joined with an agent which is capable of detec-
15   tion (e.g., a radioactive molecule or substance, such as
ricin, toxic to cells) which binds specifically to the
cell expressing the first member of the peptide pair.
Such an approach can also be used, for example, in treat-
ing hyperthyroidism. One member of the peptide pair can
20   be expressed (e.g., from a retroviral or other vector) in
the thyroid and the second member of the peptide pair
joined to radioactive iodine can be administered to an
individual in need of treatment. Pairing of the two
peptide pair members results in delivery of the radio-
25   active iodine to the thyroid; continuous delivery occurs
until the peptide pair is degraded by the body or other-
wise separated.

Members of peptide pairs can be joined to other
peptides or proteins (e.g., peptides or proteins to be
30   delivered) by chemical means or included with/incorporated
into another peptide or protein which is made by recombi-
nant DNA methods. In either case, a peptide pair member
may be joined or present at an end of the peptide or
protein or at any internal site at which the protein can
35   tolerate insertion of the peptide pair member (i.e., any

-20-

site at which the peptide pair member can be present and
not interfere with the desired function of the other
peptide or protein).

EXAMPLE 1 Assessment of the Mechanism of Specificity in
5   the Fos-Jun Heteroprotein

Peptide Synthesis and Purification

Peptides were synthesized using t-BOC chemistry on an
Applied Biosystems model 430A peptide synthesizer with
standard reaction cycles modified to include acetic an-
10 hydride capping (for a review see Kent, S.B.H., Annu. Rev.
Biochem. 57:956-989 (1988). Peptide Jun N corresponds to
residues 286-317 of the c-Jun protein (Bohmann, D. et al.,
Science 238:1386-1392 (1987); Maki, Y. et al., Proc. Natl.
Acad. Sci. USA 84:2848-2852 (1987)), and peptide Fos N
15 corresponds to residues 162-193 of the c-Fos protein (Van
Beveren, C. et al., Cell 32:1241-1255 (1983); Van
Straaten, F. et al., Proc. Natl. Acad. Sci. USA 80:3183-
3187 (1983)). Ser-295 of c-Jun and Ser-177 of c-Fos have
been replaced with tyrosine to facilitate concentration
20 determination by UV absorbance measurements. Peptide GCN4
N consists of residues 250-281 of the GCN4 protein (Hinne-
busch, A.G., Proc. Natl. Acad. Sci. USA 81:6442-6446
(1984)). All peptides have the sequence Cys-Gly-Gly
appended to the N-terminus, are acetylated at the N-termi-
25 nus, and are amidated at the C-terminus. Peptides were
cleaved by either low/high HF cleavage (Immunodynamics,
Inc., San Diego, CA) or by trifluoromethanesulfonic acid
cleavage (Kent, S.B.H., Annu. Rev. Biochem. 57:956-989
(1988) and were desalted on a Sephadex G-10 column (Pharm-
30 acia) in 5% acetic acid. Final purification was by re-
verse-phase high performance liquid chromatography (HPLC)
(Waters, Inc. and Applied Biosystems) using a Vydac pre-
parative C18 column (2.2 x 25 cm) at 25°C or 50°C. A

linear acetonitrile-H_2O gradient with segments of 0.1% to 0.2% buffer B increase per minute was used with a flow rate of 20 ml/min. Buffer A consisted of water with 0.1% trifluoroacetic acid, and buffer B consisted of 90% aceto-

5 nitrile, 10% water with 0.1% trifluoracetic acid. The identity of each peptide was confirmed by fast atom bombardment mass spectrometry (M-Scan, Inc., West Chester, PA or Mass Search, Inc., Modesto, CA) and was found to be within 1 dalton of the expected mass.

10 Circular Dichroism Studies

Circular dichroism (CD) studies were performed using a 1 cm or 1 mm cuvette (Helma or Uvonic) on an Aviv CD spectrophotometer (model 60DS or model 62DS) equipped with a thermoelectric controller. The buffer used for all CD

15 experiments except pH titrations was 50 mM NaCl, 20 mM $NaPO_4$ (pH 7.0). All peptide concentrations were determined by tyrosine absorbance (Edelhoch, H. Biochemistry 6:1948-1954 (1967)) at 275.5 nm in 5.4-6 GuHCl (Schwarz/-Mann Biotech Ultra-Pure grade) using an Aviv UV/VIS spec-

20 trophotometer (model 18DS or 14DS). The molar ellipticity at 222 nm, 0°C, was measured for the Fos and Jun N, N_{in}, N_{out}, $N_{e.d}$, $N_{e.g}$ disulfide-bonded homo- and heterodimers and for the GCN4 N homodimer. All values were found to be within the range -27,000 to -33,000 deg cm^2 $dmol^{-1}$, indi-

25 cating that the peptides are > 80% helical. Thermal melting curves were determined by monitoring the CD signal at 222 nm as a function of temperature. The pH dependence of stability was measured in 50 mM NaCl, 10 mM $NaPO_4$ at various pH values. Because the disulfide-bonded GCN4 N

30 dimer is very stable, 2 M GuHCl (Schwarz/Mann Biotech Ultra-Pure grade) was included in the buffer used for monitoring the pH dependence of stability of this peptide. The same overall shape of the pH dependence curve is obtained in the absence of GuHCl. Reversibility was

-22-

ch ck d for all thermal melts. In general, melting curves
obtained above pH 8 are not reversible, presumably because
of chemical modification and/or degradation (observed by
HPLC). Disulfide-bonded dimers that have irreversible
5 melting curves (<75% of folded signal is recovered upon
cooling) at pH 7.0 are marked (asterisk). These peptides
have undergone chemical modification and/or degradation
during the thermal melt, as judged by subsequent HPLC
analysis.

-10 Determination of the T_m

The T_m was determined by curve fitting the thermal
denaturation curve to the following equation using a
nonlinear least squares-fitting program (Kaleidagraph,
Synergy Software):

15 $$\theta = \theta_F \,(0\ K) + m_F T + [\theta_U \,(0\ K) + m_U T - \theta_F \,(0\ K) - m_F T] \left[e^{-\Delta H/RT + \Delta S/R} / 1 + e^{(-\Delta H/RT + \Delta S/R)}\right]$$

where T is temperature in K; θ is the CD signal at 222 nm;
θ_F (0 K) is the value for the CD signal of the folded
20 peptide extrapolated linearly to 0 K; m_F is the slope of
the temperature dependence of the CD signal for the folded
peptide; m_U is the slope of the temperature dependence of
the CD signal for the unfolded peptide; θ_U (0 K) is the
value for the CD signal of the unfolded peptide extra-
25 polated linearly to 0 K; ΔH is the enthalpy of unfolding
at the midpoint of the thermal denaturation curve; and ΔS
is the entropy of unfolding at the midpoint of the thermal
denaturation curve. The T_m is the temperature at which
the fraction unfolded is equal to the fraction folded (ΔG
30 = 0), and it is equal to ΔH/ΔS. The assumptions that were
made in using the equation above are as follows: thermal
melting curves are two state, described by an equilibrium
between unfolded and folded peptide; and the enthalpy and
entropy of unfolding are independent of temperature.

-23-

Because some of the Fos peptide homodimers and Fos
N_{in}-GNC4 N_{in} peptide heterodimers are not completely folded
at 0°C, the slope of the folded baseline (m_f) and the
value for the CD signal of the folded baseline extra-
polated to 0 K (θ_f (0 K)) for each of these dimers were
determined from a melting curve at pH 2.0 (conditions
where the Fos-containing peptides are more stable).

For each peptide the T_m was also determined by taking
the first derivative of the CD signal (θ) with respect to
temperature[1] (temperature in K) and finding the minimum
of this function (Cantor, C.R. and P.R. Schimmel, Bio-
physical Chemistry, W.H. Freeman, New York (1980)). All
reported values of T_m are those determined from curve
fitting. The error in the measurement of T_m (estimated
from the width of the $d\theta/d(1/T)$ plot and from repeated
curve fits starting from independent points) is ±2°C
except in cases in which 20°C>T_m > 80°C, where the error
is ±5°C. The determinations of T_m by $d\theta/d(1/T)$ and by
curve fitting agree to within the estimated errors.
Additionally, the T_m for each disulfide-bonded dimer was
measured as a function of peptide concentration (over at
least a 2.5-fold range of peptide concentration in the low
micromolar range, as estimated by the ratio of the CD
signal at low and high concentration) to determine if the
dimers were associating to higher order oligomers (O'Shea,
E.K. et al., Science 243:538-542 (1989); O'Shea, E.K., et
al., Science 245:646-648 (1989)). Peptide dimers that
were found to have a T_m that is dependent upon peptide
concentration (~3°C change in T_m over a 3- to 4-fold
concentration range), and the T_m reported is the higher of
the two measurements.

Redox Experiments

The disulfide-bonded heterodimer was incubated in
redox buffer consisting of 100-500 µM reduced glutathione

-24-

(Sigma), 100-5-- uM oxidized glutathione (Sigma), 50 mM
NaCl, 10 mM NaPO$_4$ (pH 7.4) at -23°C in an anaerobic cham-
ber (Coy Laboratory Products, Inc.). Reactions were
equilibrated at a total peptide concentration of -10-50 µM
5 for 6-16 hr and quenched under anaerobic conditions with
concentrated formic acid to a final concentration of 5% by
volume (pH < 2). The reaction products were analyzed by
microbore HPLC (Waters, Inc.) using a linear acetonitrile-
H$_2$O gradient with segments of 0.1% to 0.25% increase in
10 buffer B per minute. Analytical Vydac C-18 column (0.46 x
—   25 cm) was used at a column temperature of 25°C, 40°C or
50°C with a flow rate of 0.2 ml/min. Relative concentra-
tions of the disulfide-bonded hetero- and homodimers were
determined by integration of the corresponding peaks
15 (absorbance at 229 nm was monitored). Each redox reaction
was determined to be at equilibrium by repeating the
reaction using an equimolar mixture of reduced peptides as
the starting material. The values for ΔG_{spec} obtained from
these two different starting points agreed to within 0.1
20 kcal/mol.

RESULTS

pH Dependence of Stability in the Fos and Jun Leucine
Zipper Peptides

The relative stabilities of the Fos and Jun peptide
25 heterodimer and homodimers (Table 1) indicate that the Fos
peptide homodimer is substantially less stable than the
Fos Jun heterodimer and the Jun homodimer (O'Shea, E.K. et
al., Science, 245:646-648 (1989)); the new peptides are
shorter, lacking both an N-terminal residue and 7 amino
30 acids following the last leucine of the leucine repeat
(see Experimental Procedures). As a first step toward
identifying sources of stabilization and destabilization
in the new Fos and Jun leucine zipper dimers, the pH

dependence of the disulfide-bonded peptide pairs was
studied.

The thermal stability of the Fos and Jun homodimers
is pH dependent in a dramatic way; the T_m (melting temper-
5   ature determined from the midpoint of the thermal unfold-
ing transition, see Experimental Procedures) of the Fos
homodimer increases ~40°C from neutral to acidic pH, and
the stability of the Jun homodimer increases ~20°C from
neutral to basic pH. The stability of the heterodimer
10 changes approximately as expected from an average of the
pH dependence of the homodimers.

These large effects of pH on stability can be ex-
plained in part by examining the sequences of the Fos and
Jun leucine zippers. A particularly useful representation
15 of the sequence is the coiled-coil diagram in which the
helices of the dimers are viewed from the N-terminus with
the helix axis projecting into the page. The sequence of
the coiled-coil proteins can be divided up into positions
of the heptad repeat, labeled a-g (Hodges, R.S. et al.,
20 Cold Spring Harbor Symp. Quant. Biol., 37:299-310 (1972);
McLachlan, A.D. and M. Stewart (J. Mol. Biol. 98:293-304
(1975)). Residues at positions a and d comprise the 4-3
hydrophobic repeat characteristic of coiled coils, and
residues at positions e and g are predominantly charged
25 amino acids that can be involved in intra- or interhelical
electrostatic interactions (Hodges, R.S. et al., Cold
Spring Harbor Symp. Quant. Biol., 37:299-310 (1972);
McLachlan, A.D. and M. Stewart (J. Mol. Biol. 98:293-304
(1975)).

30   The Fos leucine zipper is very acidic, with a high
concentration of acidic amino acids at positions e, g and
b. Because each chain has a large net negative charge at
neutral pH (each chain has a net charge of -5), one ex-
pects that dimer formation would be disfavored due to
35 general electrostatic repulsion between monomers. Ad-

-26-

ditionally, the alignment of four negatively charged side
chains at position g along one face of the helix is ex-
pected to be a source of intrahelical destabilization.
The large increase in stability of the Fos homodimer upon
5   titration to low pH can, thus, be explained to result from
the relief of destabilizing intra- and interhelical elec-
trostatic interactions between acidic residues close to
the hydrophobic interface of the dimer.

The Jun leucine zipper has a slight net positive
10 charge at neutral pH (dimer has a net charge of +2 at pH
7); in addition, the concentration of charge in the Jun
leucine zipper is more spread out than in Fos. These
properties are consistent with the less dramatic increase
in stability of the Jun homodimer at high pH. Qualita-
15 tively, the pH dependence of stability for the Fos-Jun
heterodimer changes as expected from an average of the pH
dependences for the homodimers. This result suggests that
the Fos-Jun leucine zipper lacks dominant stabilizing
electrostatic interactions that are unique to the hetero-
20 dimer; in such a case, a bellshaped pH dependence curve
would be expected. In contrast, the stability of the
heterodimer increases at acidic pH values, suggesting that
intrahelical repulsion (expected from the Fos sequence) is
strong.

25   Electrostatic effects provide a possible explanation
for preferential heterodimer formation in the Fos-Jun
system. The data suggest that the peptide homodimers are
destabilized at neutral pH by residues of like charge, the
fos homodimer by acidic residues, and the Jun homodimer,
30 to a lesser extent, by basic residues. The interhelical
component of this electrostatic destabilization is re-
lieved in the heterodimer because the Fos and Jun monomers
are of opposite charge.

-27-

The Inside Residues Are Responsible for Specificity and pH-Dependent Stability

To probe the specificity of the Fos-Jun leucine zipper interaction further, structurally based hybrid
5   peptides were made by replacing portions of the Fos and Jun sequences with sequence from GCN4. A peptide corresponding to the leucine zipper region from GCN4 forms very stable homodimers (O'Shea, E.K. et al., Science 245:646-648 (1989)). As Fos and Jun are likely to fold as
10 coiled coils, the boundary between the Fos or Jun sequence and the GCN4 sequence was set by dividing the helical wheel diagram into two groups of residues: the "inside" group, consisting of the predominantly charged residues at positions e and g, and the "outside" group, consisting of
15 residues from positions b, c and f.

Two sets of hybrid leucine zipper peptides were constructed. One set of peptides has native sequence (N) from Fos or Jun at the inside positions and outside sequence from GCN4; these peptides are referred to as N_{in}.
20 The other set of peptides contains GCN4 sequence inside and Fos and Jun sequence outside; these peptides are referred to as N_{out}.

The preference for heterodimer formation was quantitated from a redox experiment in which an equimolar mixture
25 of the cysteine-containing Fos and Jun peptides is equilibrated in a redox buffer that facilitates disulfide bond formation. K_{redox} is determined from the ration of disulfide-bonded heterodimer to homodimers. The free energy of specificity for heterodimer formation (ΔG_{spec}) is equal
30 to $-RT\ln K_{redox} + RT\ln 2$. These experiments indicate that there is -2.3 kcal/mol (~100:1) preference for heterodimer in the native peptides.

The N_{in} peptides also form heterodimers preferentially, but with reduc d specificity (ΔG_{spec} is -1.2 kcal/mol).
35 The decrease in specificity of the N_{in} peptides appears to

-28-

arise from a decrease in stability of the N_{in} heterodimer;
the N_{in} heterodimer is less stable than the native hetero-
dimer but the stabilities of the N_{in} homodimers are the
same as the corresponding native homodimers (Table 1). In
5   contrast, the N_{out} peptides show essentially no specificity
(ΔG_{spec} is -0.1 kcal/mol). As expected if there is no
preference for heterodimer over homodimers, the N_{out} het-
erodimer has a stability that is intermediate between that
of the two N_{out} homodimers (Table 1). Therefore, the
10   inside residues of the Fos and Jun leucine zipper are
necessary and sufficient to mediate preferential hetero-
dimer formation.

The N_{in} peptides show pH-dependent stability that
closely resembles that of the native Fos and Jun peptides.
15   In contrast, the pH dependence for each of the N_{out} dimers
does not resemble that of the corresponding native dimer,
but resembles that of the GCN4 leucine zipper peptide.
Therefore, residues at the inside positions (positions a,
d, e and g) are also largely responsible for the pH-depen-
20   dent stability observed with the Fos and Jun peptide
dimers.

Sequence Requirements for Specificity

To investigate more thoroughly the sequence require-
ments for specificity, all possible combinations of the
25   previously described seven peptides were made (there are
28 possible combinations, and 21 of these are hetero-
dimers), and the stability of each disulfide-bonded dimer
was measured by thermal denaturation. There are large
differences in stability; the T_ms range from 17°C-38°C.
30   The free energy of specificity can be approximated by the
difference in T_m between a given heterodimer and the
average of the T_ms for the corresponding homodimers (ΔT_m =
T_m(heterodimer AB) - 1/2 [T_m(homodimer AA) + T_m(homodimer
BB)]). For the Fos-Jun peptides, ΔT_m appears to be a

quantitative measure of specificity, as it is linearly
related to the free energy of specificity, ΔG_{spec}, with a
proportionality constant of 7.4°C/kcal.

Using ΔT_m as a measure of specificity, the hetero-
5   dimeric peptide pairs can be grouped into three classes:
specificity, antispecificity and additive. The specifici-
ty classes includes peptides pairs with positive, nonadd-
itive differences in T_m ($\Delta T_m \geq +8$°C); the antispecificity
class contains peptide pairs with negative, nonadditive
10 differences in T_m ($\Delta T_m \leq -8$°C); and the additive class
consists of peptide pairs in which the stability of the
heterodimer is intermediate between that of the homodimers
(+8°C > ΔT_m -8°C).

All peptide dimers combining Jun sequence inside with
15 Fos sequence inside fall into the specificity class.
Although the mechanism of antispecificity is not apparent
at this time, all members of the antispecificity class
have Fos sequence inside combined with GCN4 sequence
inside. The other peptide combinations fall into the
20 additive class, with one exception (the Jun $N_{in \cdot Jun\ Nout}$
heterodimer falls into the specificity class, for reasons
that are not readily apparent). The most striking result
is that specific heteromdimer formation is observed with
all peptide pairs containing Fos sequence inside combined
25 with Jun sequence inside, regardless of the sequence at
the outside position, reinforcing the previous conclusion
that the inside residues (positions a, d, e and g) are the
major determinant of peptide pairing.

The inside residues consist of the predominantly
30 hydrophobic positions (a and d) and predominantly charged
positions (e and g). GCN4-based hybrid peptides contain-
ing native Fos and Jun sequence at the hydrophobic posi-
tions ($N_{a,d}$) or charged positions ($N_{e,g}$) were made to evalu-
ate the contribution of these groups of residues to speci-
35 ficity. The $N_{e,g}$ peptides form heterodimers with specific-

-30-

ity (ΔT_m and ΔG_{spec}) and stability (T_m) at least as great as
that of the native sequences (Table 1). In contrast, the
$N_{e.d}$ peptides are slightly anti-specific.

Thus, 8 residues at positions e and g of Fos and Jun

5   are sufficient to mediate preferential heterodimer for-
mation. Although the residues from Fos and Jun that
comprise the hydrophobic interface between the helices
(positions a and d) are undoubtably important for stabil-
ity (Smeal, T. et al., Genes Dev. 3:2981-2100 (1989);

10 Ransone, L.J. and I.M. Verma Annu. Rev. Cell Biol. 6:539-
557 (1990)), the residues do not appear to be important
for specificity. We conclude that van der Waals packing
differences do not have a dominant role in the discrimi-
nation between the Fos-Jun heterodimer and the corres-

15 ponding homodimers. Rather, the mechanism of specific
heterodimer formation appears to be predominantly electro-
static in nature.

This conclusion is supported further by the finding
that the $N_{e.g}$ hybrids show pH-dependent stability similar

20 to that observed with the native peptides. In particular,
the Fos $N_{e.g}$ homodimer exhibits very strong pH-dependent
stability (T_m = 41°C at pH 7 and >90°C at pH 4). In
contrast, the pH dependence of the $N_{e.d}$ hybrid dimers does
not resemble that of the native peptides. Thus, residues

25 at positions e and g in the Fos and Jun sequences also
account in large part for the dramatic pH-dependent sta-
bilities observed with the native peptides.

Discussion

The requirements for specificity in the Fos-Jun

30 system appear to be simple: 8 residues from Fos and from
Jun, in a background of the GCN4 leucine zipper, are
sufficient to mediate preferential heterodimer formation.
pH dependence studies suggest a mechanism for specificity
in which destabilization of the Fos homodimer by acidic

-31-

residues (at positions e and g) shifts the dimerization equilibrium toward the Fos-Jun heterodimer. Therefore, preferential heterodimer formation by the Fos and Jun leucine zipper peptides is largely a thermodynamic con-

5   sequence of Fos homodimer instability (O'Shea, E.K. et al., Science 245:646-648 (1989)). Destabilization of a homodimer is also used to provide specificity in the case of the tropomyosin αβ heterodimer (Lehrer, S.S. et al., Science 246:926928 (1989); Lehrer, S.S. and W.F. Stafford,

10   III, Biochemistry 30:5682-5688 (1991)).

The coupling of the ionization state of residues at positions e and g to the stability of the Fos and Jun leucine zippers can be rationalized by using the crystal structure of a peptide corresponding to the GCN4 leucine

15   zipper (O'Shea, E.K. et al., Science 254:539-544 (1991)). In this crystal structure, the methylene groups of the predominantly charged residues at positions e and g pack against the predominantly hydrophobic residues at positions a and d. Thus, the hydrophobic interface is actual-

20   ly formed by side chains from 4 residues of the heptad repeat. Additionally, terminal charged groups of residues at positions e and g of the preceding heptad are close to each other. It is likely that the close proximity of negatively charged residues at positions e and g of oppos-

25   ing Fos monomers would disrupt the complementary packing seen at the dimer interface of the coiled coil, accounting for the instability of the Fos homodimer at neutral pH.

Example 2   Synthesis of Peptide ACID-p1 and Peptide BASE-p1

30   Peptides were synthesized using small-scale FMOC HBTU reaction cycles and acetic anhydride capping on an Applied Biosystems Model 431A peptide synthesizer (for a review, see S.B.H. Kent, Annu. Rev. Biochem, 57, 957 (1988)). Peptides were cleaved from the resin with trifluoroactetic

acid (TFA) using standard cleavage methods and were de-
salted on a Sephadex G-10 column in 5% acetic acid. Final
purification was by reverse-phase high performance liquid
chromatography (HPLC) with a Vydac preparative C18 column

5   (2.2 x 25 cm) at 25°C. A linear acetonitrile-H_2O gradient
with segments of 0.1% to 0.2% buffer B increase per minute
was used with a flow rate of 10 or 20 mL/min. Buffer A
consisted of water with 0.1% TFA and buffer B consisted of
90% acetonitrile, 10% water with 0.1% TFA. Peptides were

10 >90-95% pure, as judged by analytical HPLC. The identity
of each peptide was confirmed by mass spectrometry on a
Finnegan Lasermat and was found to be within 2 daltons of
the expected mass.

Example 3   Characterization of Homodimers (ACID-p1/ACID-p1

15   **and BASE-p1/BASE-p1) and Heterodimers**

              (ACID-p1/BASE-p1)

       CD studies were performed with a 1 mm, 0.5 cm or 1 mm
cuvette on an Aviv Model 60DS or 62DS CD spectrophotometer
equipped with a thermoelecric controller. All CD studies

20 were done in the presence of phosphate-buffered saline
(PBS: 150mM NaCl, 10 mM Na phosphate, pH 7.0). All pep-
tide concentrations were determined by absorbance at 280
nm in 6 M GuHCl (26). Thermal melting curves were deter-
mined by monitoring the CD signal at 222 nm as a function

25 of temperature. Melting temperatures were estimated by
taking the first derivative of the CD signal with respect
to temperature[1] (temperature in K) and finding the mini-
mum of this function (35). Reversibility was checked for
all thermal melts and, in general, melting curves per-

30 formed at pH <8 are reversible (>90% recovery of starting
CD signal).

The ACID-p1/BASE-p1 Heterodimer is Helical and Stable

Circular dichroism (CD) spectra of ACID-p1 and BASE-
p1 at 37°C in phosphate-buffered saline (PBS), pH7 indi-
cate that the ACID-p1 and BASE-p1 peptides are predomi-
5 nantly unfolded). In contrast, the characteristic the
helical minima at 222 and 208 nm in the spectrum of an
equimolar mixture of ACID-p1 and BASE-p1 indicate that the
mixture is highly helical. At 0°C, a 100 uM mixture of
ACID-p1 and BASE-p1 is - 100% helical($(\theta)_{222}$ = -34,000).
10 The helical structure formed by the ACID-p1 and BASE-p1
mixture is stable, as it undergoes a cooperative unfolding
transition when denatured with urea. In contrast, the
isolated peptides show no evidence for cooperative unfold-
ing. Sedimentation equilibrium studies of ACID-p1 and
15 BASE-p1 at 20°C in PBS, pH 7 indicate that this mixture
is heterodimeric. Thus, the ACID-p1 and BASE-p1 peptides
associate preferentially and fold as a stable, helical
heterodimer.

The amide proton exchange behavior of the ACID-p1 and
20 BASE-p1 heterodimer was characterized by nuclear magnetic
resonance (NMR). One-dimensional NMR spectra were re-
corded on a Bruker AMX 500 MHz spectrometer at 20°C with a
sweep width of 6024.1 Hz and a recycle time of 1.2 sec.
8192 points were collected and water was suppressed by
25 continuous irradiation. Trimethylsilylpropionic acid was
used as a standard (A. DeMarco, J. Magn. Reson. 26, 527
(1977)). Data were processed with the FTNMR software
package (proviced by Dr. Dennis Hare, Hare Research, Inc.)
and spectra were apodized with a gaussian window. Since
30 the global stability of the ACID-p1 and BASE-p1 hetero-
dimer under the exchange conditions is -6.7 kcal/mol, the
expected maximum degree of protection from exchange is -
10^5. At a time when protons that are protected by a
factor of -10^3 are expected to be half exchanged, many
35 proton resonances have full intensity. Additionally, at a

-34-

later time point corresponding to a protection of 10^4,
twelve amide proton resonances are present in the spec-
trum.   Therefore, the ACID-p1 and BASE-p1 heterodimer has
slowly-exchanging amide protons with protection factors
5 near those expected from the global stability of the
heterodimer, suggesting that the molecule has a well-
packed 3^0 interface.

Characterization of the Disulfide-Bonded Heterodimer and Homodimers

10   Our design predicts that the ACID and BASE peptides
will fold as parallel, helical dimers, but at reasonable
peptide concentrations the isolated ACID-p1 and BASE-p1
are too unstable to study.   One way to stabilize dimeric
coiled-coil peptides is to join the peptides with a flex-
15 ible disulfide bond.   Therefore, versions of the ACID and
BASE peptides (ACID-p1n and BASE-p1n) containing an N-
terminal cysteine followed by two glycines were synthe-
sized (glycines were added to allow disulfide bond for-
mation without the distortion of the coiled-coil struc-
20 ture.)   CD studies demonstrate that the disulfide-bonded
heterodimer and homodimers are > 80% helical at 0^0C and
that each dimer shows a cooperative thermal uinfolding
transition indicating that the ACID and BASE peptides can
be stabilized sufficiently with a disulfide bond to permit
25 folding as stable, helical dimers.

        The concentration dependence of stability was studied
for each of the disulfide-bonded peptide dimers to deter-
mine if the orientation of the helices is parallel and if
the dimers associate to a higher-order oligomers.   When
30 the ACID-p1N and BASE-p1N heterodimer and homodimer are
joined in the parallel oreintation, the stabi;lity of each
dimer is independent of peptide concentration over a range
of concentration from ~2.5 uM to 170 uM.   In contrast, a
heterodimer disulfide-bond d in the antiparallel prientat-

ion has a CD signal that is dependent on peptide con-
centration, indicating that the antiparallel dimers are
associating to higher-order oligomers. The antiparallel
heterodimer consists of the peptide ACID-p1N (N-terminal
5 Cys-Gly-Gly) disulfide bonded to a BASE peptide that has a
C-terminal sequence of Gly-Gly-Cys. In PBS containing
2.25 M GuHCl at 0°C, a 0.9 μM sample of the antiparallel
dimer has a $[\theta]_{222}$ value of -11,080 and a 24.5 μM sample
has a $[\theta]_{222}$ value of -25,300. In contrast, the helicity
10 of the parallel, disulfide-bonded ACID and BASE hetero-
dimer and homodimers is independent of peptide concen-
tration over a sample concentration range from -2.5 μM to
170 μM.   Collectively, these experiments indicate that
the ACID and BASE peptides are parallel and dimeric.

15 **Mechanism of Specificity**

Our design strategy sought to drive preferential
heterodimer formation by destabilizing the homodimers
rather than by stabilizing the heterodimer. If eletro-
static destabilization of the homodimers is occurring, the
20 homodimers will be unstable in conditions of neutral pH
and low ionic strength and will become more stable when
the charges are titrated at extremes of pH or when the
ionic strength is increased. In contrast, if stabili-
zation of the heterodimer by ion pairs is present, the
25 heterodimer will be most stable at neutral pH and will
become less stable as the charge on residues involved in
ionic interactions is titrated at pH extremes. Thus, a
bell-shaped pH dependence of stability curve would be
observed. Additionally, if electrostatic stabilization is
30 important, the heterodimer will be most stable at low
ionic strength and will become less stable as residues
involved in ionic pairs are shielded by ions.

The increased stability of the disulfide-bonded
species allowed us to investigate the pH and ionic

-36-

strength dependence of stability of the homodiemrs. The
stability of both disulfide-bonded homodimers is strongly
pH and ionic strength dependent. The ACID-p1N homodimer
is > 80% more stable at acidic pH than at pH 7, demon-
5   strating clearly that it is destabilized by acidic resi-
dues at neutral pH. Similarly, the BASE-p1N homodimer is
destabilized at neutral pH by positively charged basic
residues, as it becomes -20°C more stable as the pH is
changed from neutral to basic pH. Additionally, the di-
10 sulfide-bonded homodimers are stabilized by increasing
ionic strength. These results indicate that the homo-
dimers are destabilized by unfavorable electrostatic
interactions.

Interestingly, at neutral pH the stability of the
15 BASE-p1N homodimer is much greater than that of the ACID-
p1N homodimer. It is likely that part of the reason for
the greater stability at neutral pH of the BASE-p1N homo-
dimer as compared to the ACID-p1N homodimer is the differ-
ence in the length of the sidechains of residues at posi-
20 tions e and g. Whereas ACID-p1N has glutamate (containing
two methylene groups) at position e and g, BASE-p1N has
lysine (four methylene groups). The longer lysine side-
chain allows for more flexibility and solvation of the
terminal charged group. The idea that the length of
25 sidechains at positions e and g is important in
determining homodimer stability is supported by studies of
BASE peptides containing the non-natural amino acids
ornithine (three methylene groups) or diaminobutyric acid
(two methylene groups). BASE homodimers containing these
30 non-natural amino acids are predominantly unfolded and the
stability of the corresponding ACID/BASE heterodimer
decreases with the decreasing sidechain length. Two
analogues of the BASE-p1N peptide were studied; one con-
taining diamobutyric acid at positions e and g (DAB-p1N),
35 and the other containing ornithine at these positions

-37-

(ORN-p1N). Although the disulfide-bonded BASE-p1N homo-
dimer (containing lysine at positions e and g) has a T_m of
66°C, neither the disulfide-bonded DAB-p1N homodimer nor
the ORN-p1N homodimer shows evidence for structure at 0°C
5 or evidence for a cooperative thermal unfolding trans-
ition. Additionally, the disulfide-bonded heterodimers
formed between ACID-p1N and DAB-p1N or ORN-p1N have T_m's
of ~63°C ad ~80°C, respectively (the ACID-p1N/BASE-p01N
heterodimer has a T_m >100°C).

10   In contrast to the stability of the homodimers, the
stability of the heterodimer is relatively independent of
ionic strength and pH. Because the disulfide-bonded ACID-
p1N/BASE-p1N heterodimer cannot be thermally denatured in
the absence of chemical denaturant, the pH and ionic
15 strength dependence of the ACID-p1 + BASE-p1 mixture was
studied in the absence of a disulfide bond. From pH 4 to
pH 9 the stability of the ACID-p1 + BASE-p1 heterodimer is
relatively independent of pH. It is difficult to assess
the stability of the heterodimer at pH extremes (3.5 > pH
20 > 10.5) because the ACID-p1 + BASE-p1 thermal denaturation
curve contains more than one transition, suggesting that
homodimers are populated significantly. It is puzzling
that the heterodimer does not exhibit pH and ionic
strength dependent stability, given that the heterodimer
25 has charged sidechains positioned to be able to form salt
bridges (of the same type seen in the GCN4 leucine zipper
structure). One possible explanation for the lack of pH
and ionic strength dependent stability is that salt bridg-
es form in the heteordimer but they are not significantly
30 stabilizing. There is precedent for this explanation, as
the GCN4 leucine zipper peptides show little pH and ionic
strength dependent stability even though salt bridges are
seen in the crystal structure. Alternatively, the stabi-
lizing effect of salt bridges could be coincidentally
35 offset by destabilizing intrahelical repulsion. It is

-38-

plausible that such compensating effects exist, as the
Fos/Jun heterodimer is more stable at low pH than neutral
pH. The greater stability at low pH suggests that there
is substantial intrahelical repulsion at neutral pH. In
5   any case, these studies of pH and ionic stength dependence
suggest that destabilization of the homodimers makes a
major contribution to preferential heterodimer formation.

Quantitation of Specificity
        Because the preference for heterodimer in the ACID
10 and BASE peptides is so large, the ratio of heterodimer to
homodimer cannot be measured readily from an equilibrium
mixture of the two peptides. However, the degree of
specificity can be estimated because K_{spec} (the equilibrium
constant describing the ratio of heterodimer to homodimer)
15 is linked thermodynamically to the dissociation constants
for each of the dimers. Therefore, the dissociation
constants for each dimer were determined so that the
degree of specificity, ΔG_{spec} ($= -RT \ln K_{spec}$), could be esti-
mated. The following quantitation of specificity should
20 be considered an estimate because the linkage relationship
relating the dissociation constants relies upon the as-
sumption that the monomer-dimer equilibria are two-state.
        The dissociation constant for the ACID-p1N and BASE-
p1N heterodimer was determined by monitoring the CD signal
25 as a function of urea concentration. These resulting
denaturation curve was fit to a two-state model for mono-
mer-dimer equilibrium to obtain a dissociation constant
for the heterodimer of 3×10^{-8} M. A two-state model for
unfolding was assumed and the urea denaturation curve for
30 the heterodimer was fit with a non-linear least squares
fitting program (KaleidaGraph, Synergy Software) to the
following equation that describes the GuHCl dependence of
the CD signal for a monomer-dimer equilibrium: $\theta = \theta d +$

$$(\theta_m - \theta d) \; [-e^{(-\Delta G^\circ + m[GuHCl])/RT}/4C_T + ((e^{(-\Delta G^\circ + m[GuHCl])/RT})^2/16C_T^2 + e^{-(-\Delta G^\circ + m[GuHCl])/RT}/2C_T)^{1/2}],$$

where θ = CD signal at 222 nm; θ_d = guanidine dependence
of the CD signal of the dimer; θ_m = guanidine dependence
of the CD of the monomer; ΔG° = free energy of folding
under standard state conditions of 20°C and 1 M concen-
tration of peptide chains; m = slope of the guanidine
dependence of ΔG; R = gas constant; T = temperature in K;
C_T = total concentration of peptide chains. From this
fit, a dissociation constant of 3 x 10^{-8} was obtained. An
estimate of the dissociation constant for each of the
homodimers was obtained from measurements of the helical
CD signal as a function of peptide concentration. The
dissociation constant for the BASE-p1 homodimer is ~1 x
10^{-3} M and that for the ACID-p1 homodimer is > 5 x 10^{-3} M.
These dissociation constants place a lower limit on the
degree of preference for heterodimer, ΔG_{spec}, of -6.5
kcal/mol (> 10^5-fold preference for heterodimer).

Specificity can also be estimated by measuring the
difference between the melting temperature (T_m) of the
heterodimer and the average of the T_ms (melting tempera-
ture) for the homodimers (ΔT_m). ΔT_m has been measured for
other disulfide-bonded leucine zipper peptides and has
been shown to be related to ΔG_{spec} by a proportionality
constant of 7.4°C/kcal mol^{-1}. ΔT_m for the disulfide-bonded
ACID-p1N and BASE-p1N peptides is > 56°C. If the same
proportional relationship between ΔT_m and ΔG_{spec} exists with
the ACID and BASE peptides, this lower limit for ΔT_m
implies that ΔG_{spec} is at least -7.5 kcal/mol (>$10^{5.6}$-fold
preference for the heterodimer).

Thus, the heterodimer is preferred over the ACID-p1
and BASE-p1 homodimers by at least ~10^5-fold. This degree
of specificity is much greater than that observed for the
Fos and Jun peptides where the Fos/Jun heterodimer is
preferred by only ~10^2-fold.

-40-

Equivalents

Those skilled in the art will recognize, or be able
to ascertain using not more than routine experimentation,
many equivalents to the specific embodiments of the inven-
5   tion described herein.   Such equivalents are intended to
be encompassed by the following claims.

-41-

CLAIMS

1. A synthetic peptide consisting essentially of 30
    amino acid residues, wherein the amino acid residues
    are as follows:
    a) amino acid residues 4, 11, 18, 25, 6, 13, 20 and
        27 are negatively charged amino acid residues;
    b) amino acid residues 7, 14, 21, 28, 3, 10, 17 and
        24 are hydrophobic amino acid residues;
    c) amino acid residues 1, 2, 8, 15, 22, 29, 9, 16,
        23 and 30 are small, uncharged amino acid resi-
        dues; and
    d) amino acid residues 5, 12, 19 and 26 are charged
        amino acid residues.

2. The synthetic peptide of Claim 1 wherein:
    a) the amino acid residues of (a) are glutamate
        residues;
    b) the amino acid residues of (b) are leucine resi-
        dues or aspartate residues;
    c) the amino acid residues of (c) are alanine resi-
        dues or glutamine residues; and
    d) the amino acid residues of (d) are glutamate
        residues, lysine residues or tryptophan resi-
        dues.

-42-

3. A synthetic peptide consisting essentially of 30
   amino acid residues, wherein the amino acid residues
   are as follows:
   a)   amino acid residues 4, 11, 18, 25, 6, 13, 20 and
         27 are positively charged amino acid residues;
   b)   amino acid residues 7, 14, 21, 28, 3, 10, 17 and
         24 are hydrophobic amino acid residues;
   c)   amino acid residues 1, 2, 8, 15, 22, 29, 9, 16,
         23 and 30 are small, uncharged amino acid resi-
         dues; and
   d)   amino acid residues 5, 12, 19 and 26 are charged
         amino acid residues.

4. The synthetic peptide of Claim 3 wherein:
   a)   the amino acid residues of (a) are lysine resi-
         dues;
   b)   the amino acid residues of (b) are leucine resi-
         dues or aspartate residues;
   c)   the amino acid residues of (c) are alanine resi-
         dues or glutamine residues; and
   d)   the amino acid residues of (d) are glutamate
         residues, lysine residues or tryptophan resi-
         dues.

-43-

5. A helical heterodimer consisting essentially of two
    synthetic peptides, designated peptide ACID-pl and
    peptide BASE-pl, wherein:

    a)   peptide A consists essentially of 30 amino acid
         residues, wherein the amino acid residues are as
         follows:

         (1)   amino acid residues 4, 11, 18, 25, 6, 13,
               20 and 27 are negatively charged amino acid
               residues;

         (2)   amino acid residues 7, 14, 21, 28, 3, 10,
               17 and 24 are hydrophobic amino acid resi-
               dues;

         (3)   amino acid residues 1, 2, 8, 15, 22, 29, 9,
               16, 23 and 30 are small, uncharged amino
               acid residues; and

         (4)   amino acid residues 5, 12, 19 and 26 are
               charged amino acid residues

    b)   peptide B consists essentially of 30 amino acid
         residues, wherein the amino acid residues are as
         follows:

         (1)   amino acid residues 4, 11, 18, 25, 6, 13,
               20 and 27 are positively charged amino acid
               residues;

         (2)   amino acid residues 7, 14, 21, 28, 3, 10,
               17 and 24 are hydrophobic amino acid resi-
               dues;

         (3)   amino acid residues 1, 2, 8, 15, 22, 29, 9,
               16, 23 and 30 are small, uncharged amino
               acid residues; and

         (4)   amino acid residues 5, 12, 19 and 26 are
               charged amino acid residues.

-44-

6.   The helical h terodimer of Claim 5 wherein:
  a)   in peptide ACID-p1:
     (1)   the amino acid residues of (a) are gluta-
           mate residues;
     (2)   the amino acid residues of (b) are leucine
           residues or aspartate residues;
     (3)   the amino acid residues of (c) are alanine
           residues or glutamine residues; and
     (4)   the amino acid residues of (d) are gluta-
           mate residues, lysine residues or tryp-
           tophan residues.
  b)   in peptide BASE-p1:
     (1)   the amino acid residues of (a) are lysine
           residues;
     (2)   the amino acid residues of (b) are leucine
           residues or aspartate residues;
     (3)   the amino acid residues of (c) are alanine
           residues or glutamine residues; and
     (4)   the amino acid residues of (d) are gluta-
           mate residues, lysine residues of tryp-
           tophan residues.

7.   A coiled-coil heterodimer comprising at least one
pair of synthetic peptides, represented by the fol-
lowing helical wheel:
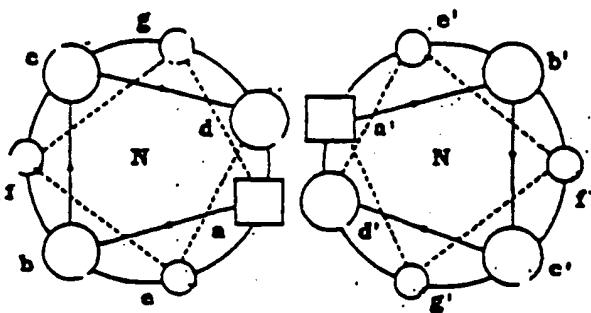
wherein the first member of the peptide pair is
designated peptide ACID-p1 and the second member of
the peptide pair is designated peptide BASE-p1 and:

a)   the amino acid residues at positions e and g of
peptide A are negatively charged amino acid
residues;

b)   the amino acid residues at positions e' and g'
of peptide B are positively charged amino acid
residues;

c)   the amino acid residues at positions a and d of
peptide A and positions a' and d' of peptide B
are hydrophobic amino acid residues;

d)   the amino acid residues at positions b and c of
peptide A and positions b' and c' of peptide B
are uncharged amino acid residues; and

e)   the amino acid residues at position f of peptide
A and position f' of peptide B are charged amino
acid residues.

-46-

8. The coiled-coil helical heterodimer of Claim 7 where-
    in:

    a) the amino acid residues of (a) are glutamate
        residues;

5   b) the amino acid residues of (b) are lysine resi-
        dues;

    c) the amino acid residues of (c) are leucine resi-
        dues;

    d) the amino acid residues of (d) are alanine resi-
_10_   dues or glutamine residues; and

    e) the amino acid residues of (e) are glutamate
        residues or lysine residues.

9. A multi-unit peptide comprising at least two synthe-
    tic peptides of Claim 1.

15 10. A multi-unit peptide comprising at least two synthe-
         tic peptides of Claim 3.

11. A coiled-coil heterodimer of Claim 7, further com-
     prising more than one pair of synthetic peptides
     ACID-p1 and BASE-p1.

20 12. A chimeric peptide comprising a synthetic peptide of
         Claim 1 and a second peptide which is not a synthetic
         peptide of Claim 1.

13. A chimeric peptide of Claim 12, wherein the second
     component is selected from the group consisting of:
25   peptides, polypeptides, glycopeptides, small organic
     molecules and detectable labels.

14. A chimeric peptide of Claim 13, which is detectably
     labeled.

-47-

15. A multi-unit peptide comprising at least two chimeric synthetic peptides, each chimeric synthetic peptide comprising at least one synthetic peptide of Claim 1 and a second component which is not a synthetic
5 peptide of Claim 1.

16. A multi-unit peptide of Claim 15, wherein the second component is selected from the group consisting of: peptides, polypeptides, glycopeptides, small organic molecules and detectable labels.

10 17. A multi-unit peptide of Claim 16 which is detectably labeled.

ACID-p1   **BASE-p1**
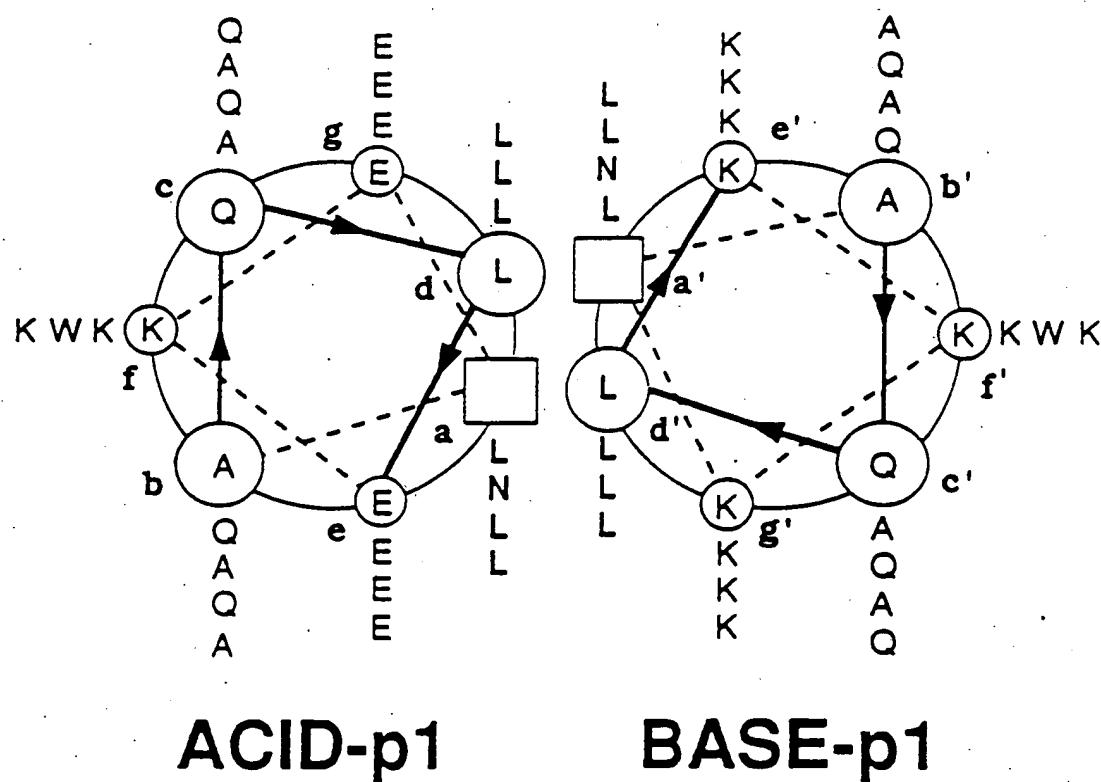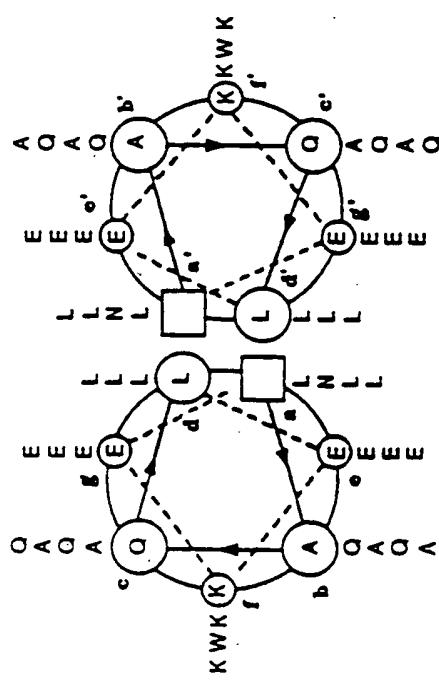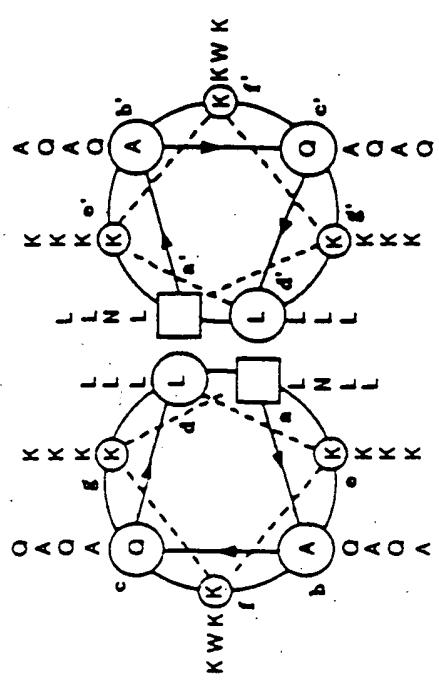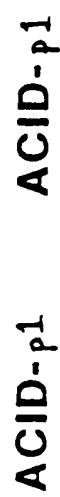
FIGURE 1

FIGURE 2A


FIGURE 2B
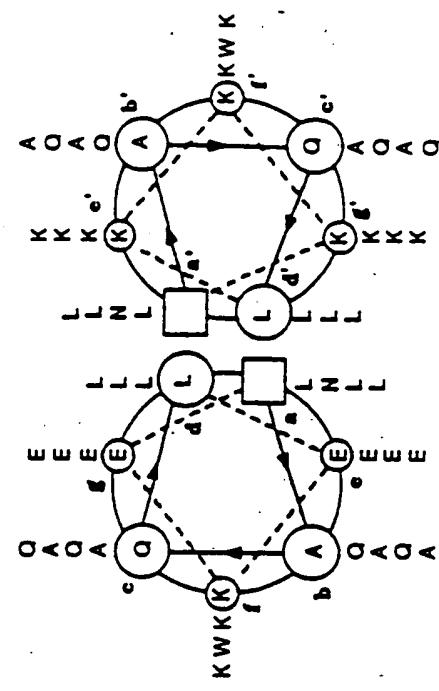

FIGURE 2C
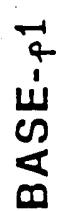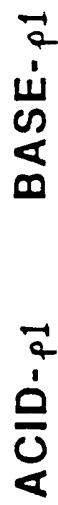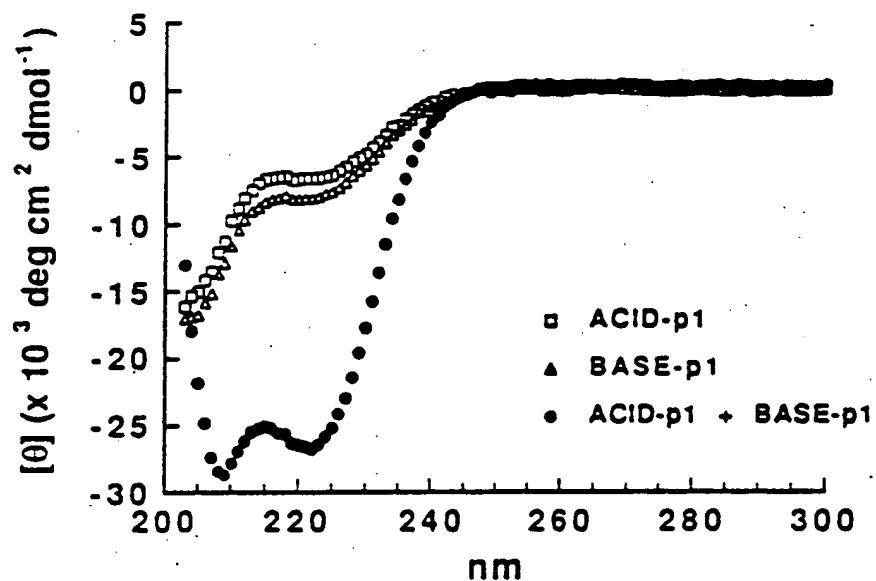
FIGURE 3

FIGURE 4
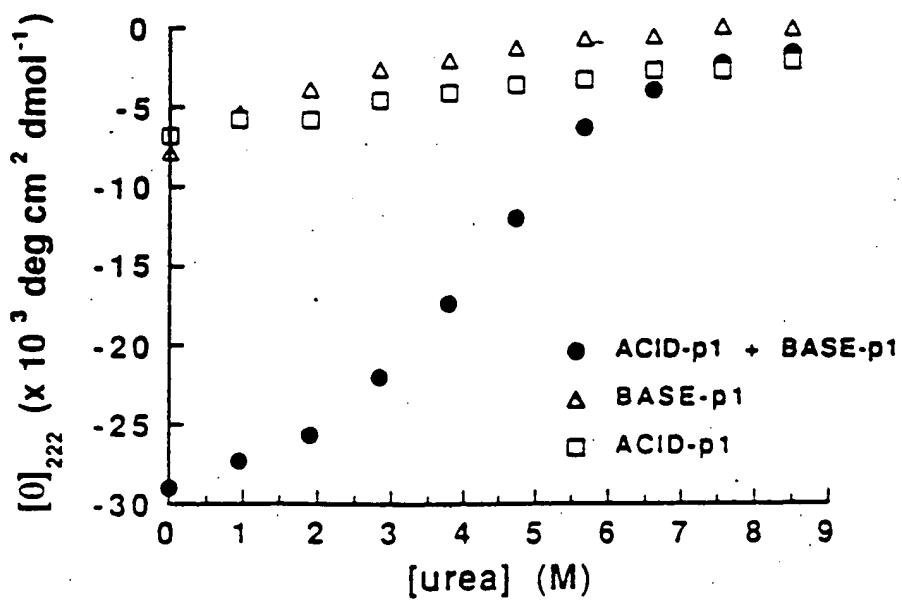
INTERNATIONAL SEARCH REPORT

International Applicat

PCT/US 93/C0884

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K7/10

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
Int.Cl. 5	C07K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 256, no. 3, 10 February 1981, BALTIMORE, MD US pages 1214 - 1224 R. S. HODGES ET AL. 'Synthetic Model for Two-stranded alpha-Helical Coiled-coils' cited in the application see part of 'Results and Discussion' on pages 1215-1218 see figure 1; table 1 --- -/--	1-11

* Special categories of cited documents : [10]

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

'&' document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 JUNE 1993	2 8 -06- 1993

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	FUHR C.K.B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT	(CONTINUED FROM THE SECOND SHEET)	
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 259, no. 21, 10 November 1984, BALTIMORE, MD US pages 13253 - 13261 S.Y.M. LAU ET AL. 'Synthesis of a Model Protein of Defined Secondary and Quaternary Structure' cited in the application see page 13259, right column, paragraph 2 - page 13260, right column, paragraph 2; figure 2; table 1 ---	1-11
P,A	WO,A,9 210 567 (CREATIVE BIOMOLECULES, INC.) 25 June 1992 see page 13, paragraph 1 - page 14, paragraph 1; claims 1-15 -----	1-15

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.   15/06/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9210567	25-06-92	US-A- 5197973	30-03-93
		AU-A- 9132391	08-07-92